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Polyclonal and Monoclonal Antibodies Specific to the Chrysanthemic Acid Moiety of Pyrethroid Insecticides

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Abstract: Two polyclonal and two monoclonal antibodies raised against chrysanthemic acid (CAA) were prepared for ELISA of pyrethroid insecticides with the common CAA moiety. The monoclonal antibody KCA226 showed the highest reactivity towards allethrin among the antibodies in C-ELISA, although KCA226 was more sensitive to higher concentrations of methanol than the polyclonal antibodies in ELISA. KCA226 was reacted linearly with allethrin at concentrations ranging from 1 to 10 μg litre^{-1} in 0·5% methanol and from 300 to 10 000 μg litre^{-1} in 30% methanol. KCA226 reacted specifically with the pyrethroid insecticides with the CAA moiety but was much less reactive with CAA itself. These results suggest that C-ELISA based on KCA226 is useful for the assay of pyrethroid residues with the common CAA moiety. © 1998 Society of Chemical Industry

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Key words: monoclonal antibodies; immunoassay; ELISA; pyrethroid; insecticide; chrysanthemic acid

1 INTRODUCTION

Natural pyrethrins prepared from dried Chrysanthemum flowers are used to control insect pests in stored foods, and household and industrial pests, because of their safety and rapid action. However, a major disadvantage of natural pyrethrins, especially for the control of agricultural pests, lies in their instability in the presence of air and light. Natural pyrethrins contain four main insecticidal components, of which the most active, pyrethrin I, is the ester of pyrethrolone and chrysanthemic acid. The synthesis of chrysanthemic acid opened up the possibility of synthetic pyrethroids, the first of which was allethrin.[1] Thereafter, a number of synthetic pyrethroids with the chrysanthemic acid moiety were developed and used for the control of household and agricultural insect pests.[2]

The monitoring of pyrethroid residues is very important for safety assessment of foods and the environment, as well as for human health care. Recently, immunoassays for the detection of pyrethroid compounds have been developed for bioallethrin,[3,4] permethrin/phenothrin,[5,6] bioresmethrin[7] and etofenprox.[8] The antibodies used for these assays were produced by the immunization of animals with the hapten conjugates derived from the corresponding parent compounds and they were almost specific to each of the pyrethroids.

Since pyrethroid compounds, such as allethrin, resmethrin, phenothrin and tetramethrin, have the common chrysanthemic acid (CAA) moiety in their

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structures, we attempted to prepare monoclonal antibodies specific to the CAA moiety. Antibodies prepared were examined for cross-reactivity towards a number of pyrethroid compounds with and without the CAA moiety in competitive enzyme-linked immunosorbent assay.

2 EXPERIMENTAL METHODS

2.1 Chemicals and biochemicals

CAA was obtained from Sumitomo Chemical (Japan). The pyrethroid compounds used in this study were: allethrin, bioallethrin, cyhalothrin, cypermethrin, fenpropathrin, permethrin, phenothrin, pyrethrins, resmethrin and tetramethrin (Riedel-de Haën, Germany) and cycloprothrin, cyfluthrin, deltamethrin, etofenprox, flucythrinate and tau-fluvalinate (Wako Pure Chemical, Japan).

The biochemicals were: ovoalbumin (OVA; Wako Pure Chemical), bovine serum albumin (BSA; Sigma Chemical, USA) and peroxidase (POD)-conjugated anti-mouse IgG antibodies and POD-conjugated anti-rabbit IgG antibodies (Organon teknika, USA). Microplates with 96 wells used for cell culture and microtiter plates with 96 wells used for enzyme-linked immunosorbent assay (ELISA) and competitive ELISA (C-ELISA) were purchased from Corning (USA). All other chemicals and reagents were of analytical grade.

2.2 Preparation of hapten–protein conjugates

The hapten CAA was coupled covalently with OVA and with BSA by the mixed-anhydride method.[9] CAA (150 μmol) was reacted with OVA (80 mg) or BSA (80 mg). After reaction, the mixture, including CAA coupled with the protein, was dialysed against phosphate-buffered saline overnight at 4°C. The solution was used for further examination as a conjugate of CAA and OVA (CAA-OVA) or a conjugate of CAA and BSA (CAA-BSA).

2.3 Preparation of polyclonal antibodies

Two eight-week-old female white rabbits (weight about 1·0 kg) were each immunized with CAA-OVA (1·0 mg) as previously described.[10] The sera raised were used for further examinations as polyclonal antibodies.

2.4 Preparation of monoclonal antibodies

Five eight-week-old female Balb/C mice (weight about 25 g) were each immunized with CAA-OVA (100 μg) and their splenocytes prepared were fused with P3-X63-AG8.653 myeloma cells[11] as previously described.[10] To obtain monoclonal antibodies reacted with pyrethroid compounds, cultured fluids of hybridomas were first screened for their binding ability to the antigen CAA-BSA in ELISA. The cultured fluids selected were then screened again for reaction with pyrethrins (10 mg litre^{-1}) in C-ELISA. Positive hybridomas were cloned by limiting dilution, and their cultured fluids were taken for further examination as monoclonal antibodies.

2.5 ELISA

ELISA was carried out according to the procedure as previously described.[10] Microtiter plates with 96 wells were coated with 50 μl of the antigen CAA-BSA (1 mg litre^{-1}) needed to saturate the available sites on the wells. Aliquots (100 μl) of each of polyclonal and monoclonal antibodies diluted to 70–80% of the maximal reaction (determined by pre-titration) with borate-buffered saline (BBS; borate, 85 mM, NaCl, 150 mM, pH 8·0), supplemented with an appropriate concentration of methanol (0·5 to 70%; methanol + BBS, 0·05 + 9·95 to 7 + 3 by volume) and 0·3% BSA, were then added to wells coated with CAA-BSA. The plates were incubated for 1 h at 25°C, followed by the addition of the POD-conjugated anti-rabbit or anti-mouse IgG antibodies. After washing, the absorbance at 450 nm developed by colour development system using 3,3′,5,5′-tetramethylbenzidine was measured in an automated microplate reader (Bio-tek Instruments, USA). This could measure the absorbance linearly in the range 0·0 to 3·3 OD units.

All samples were assayed in duplicate and the assay was also performed in triplicate.

2.6 C-ELISA

C-ELISA used for assay of pyrethroid compounds was also carried out according to the procedure as previously described.[10] Analytes were each dissolved in methanol and a methanol solution was mixed with BBS supplemented with 0·3% BSA at a final concentration of 0·5% or 30% methanol (methanol + BBS, 0·05 + 9·95 or 3 + 7 by volume). These samples were added to wells coated with the antigen CAA-BSA and were immediately mixed with equal volumes of antibodies diluted to twice the concentrations of the above ELISA (Section 2.5) with the same buffer and incubated for 1 h at 25°C. The plate was then treated by the same steps as with ELISA.

On assay under different pH conditions, 85 mM phosphate buffer (pH 5·0, 6·0 and 7·0), 85 mM borate buffer (pH 8·0) and 85 mM Tris-Cl buffer (pH 9·0) were used for dilutions of the monoclonal antibody and analyte instead of the above borate buffer (pH 8·0). These

buffers were also supplemented with 150 mM NaCl, 0·3% BSA and 0·5% methanol.

All samples were assayed in duplicate and the assay was also performed in triplicate.

3 RESULTS

3.1 Polyclonal and monoclonal antibodies specific to the CAA moiety

Two polyclonal antibodies, poly A and poly B, were prepared from two rabbits immunized with CAA-OVA, respectively. The titer of both antibodies was found to be sufficient, since the dilution rate for 50% of the maximum reactivity with the antigen CAA-BSA in ELISA was about 200 000-fold with poly A and about 100 000-fold with poly B.

Monoclonal antibodies were also produced by the use of the same immunogen as with the polyclonal antibodies, but careful screening was carried out to select one reacting with pyrethroid compounds with the CAA moiety. When hybridomas were cultured in the microplates with 96 wells, viable colonies were formed on wells after 10 to 14 days. Then, monoclonal antibodies secreted in the cultured fluids were screened for binding ability with the antigen CAA-BSA in ELISA. By this screening, 80 out of 783 wells with colonies were selected as positive wells. Next, the wells were rescreened for inhibition by pyrethrins in C-ELISA. The reaction of two out of 80 wells was inhibited competitively by addition of pyrethrins (10 mg litre^{-1}).

The cloned hybridomas were cultured in complete medium and their culture fluids were used as the monoclonal antibodies KCA190 and KCA226. KCA190 and KCA226 were characterized as IgG2b and IgG2a, respectively, by means of ELISA using a biotinylated anti-mouse Ig subclass collection (Amersham, UK).

3.2 Effects of methanol on the reaction of antibody with the antigen CAA-BSA

Since pyrethroid compounds are poorly soluble in water, the residues are often extracted with an organic solvent prior to analysis. The effects of some organic solvents on reaction between the antigen CAA-BSA and antibody in ELISA were therefore examined. When acetone, acetonitrile, dimethylsulfoxide or methanol was added to dilution buffers of poly A, poly B, KCA190 or KCA226, methanol showed the least adverse effects on the reaction (data not shown).

The effects of methanol were different among the four antibodies, as shown in Fig. 1. Although the reactivities of poly A and poly B were reduced drastically by adding 70% of methanol, both polyclonal antibodies tolerated 60% methanol. On the other hand, the reactivities of both monoclonal antibodies were more sensitive to methanol than those of the polyclonal antibodies, although the reactivity of both monoclonal antibodies was maintained up to 40% methanol.

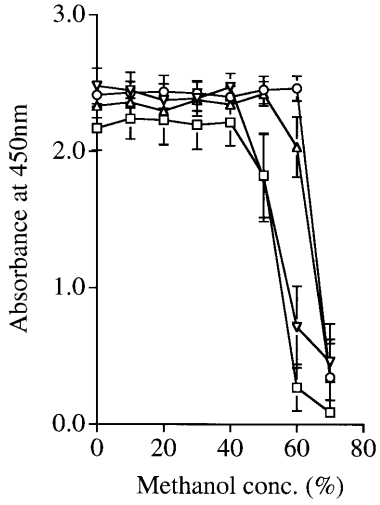

Fig. 1. The effects of methanol on the reaction of antibodies with the antigen CAA-BSA: (○) poly A, (△) poly B, (▽) KCA190 and (□) KCA226. Results are represented as means ± standard deviation (n = 3).

Based on these results, we selected 0·5% and 30% methanol under the assay conditions for further analysis in C-ELISA.

3.3 Reactivity of the antibodies with allethrin

We compared the reactivities of four antibodies with the pyrethroid allethrin, since the natural pyrethrins are a mixture of four main insecticidal compounds.

The reactivities of the antibodies with allethrin were compared in C-ELISA containing 0·5% methanol. As shown in Fig. 2A, both polyclonal antibodies showed linear inhibition curves at concentrations ranging from

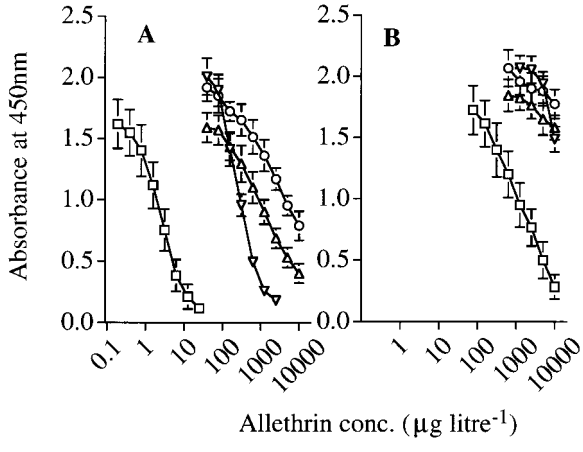

Fig. 2. The reactivity of polyclonal and monoclonal antibodies with allethrin in (A) 0·5% methanol or (B) 30% methanol: (○) poly A, (△) poly B, (▽) KCA190 and (□) KCA226. Results are represented as means ± standard deviation (n = 3).

TABLE 1
The Cross-Reactivity of KCA226 with Pyrethroid Compounds and the CAA Moiety in C-ELISA

Compound	IC_{50} ($\mu g\ litre^{-1}$) ($\pm SD$)[a]	
	0·5% methanol	*30% methanol*
Allethrin	3·2 (±0·8)	2200 (±200)
Bioallethrin	7·1 (±0·8)	2400 (±100)
Pyrethrins	9·4 (±2·3)	2600 (±200)
Tetramethrin	2·8 (±0·9)	700 (±70)
Phenothrin	91 (±18)	>10000
Resmethrin	96 (±37)	>10000
Cyfluthrin	1400 (±100)	>10000
Cypermethrin	6000 (±2800)	>10000
Permethrin	1100 (±100)	>10000
Deltamethrin	>10000	>10000
Cycloprothrin	>10000	>10000
Cyhalothrin	>10000	>10000
Etofenprox	>10000	>10000
Fenpropathrin	>10000	>10000
Flucythrinate	>10000	>10000
Tau-fluvalinate	>10000	>10000
CAA	>10000	>10000

[a] $n=3$.

100 to 10000 μg litre^{-1}. The 50% inhibition concentration (IC_{50}) value of allethrin for both polyclonal antibodies was about 1000 μg litre^{-1}. On the other hand, the reactivities of both monoclonal antibodies with allethrin were higher than those of the polyclonal antibodies. The inhibition curves of both monoclonal antibodies were linear at concentrations ranging from 100 to 1000 μg litre^{-1} for KCA190 and from 1 to 10 μg litre^{-1} for KCA226. Thus, the range of the reaction of the monoclonal antibodies with allethrin was narrower than that of the polyclonal antibodies. The IC_{50} values of allethrin for KCA190 and KCA226 were 500 μg litre^{-1} and 3 μg litre^{-1}, respectively. Therefore, it was found that, of four antibodies examined, KCA226 was the most reactive with allethrin.

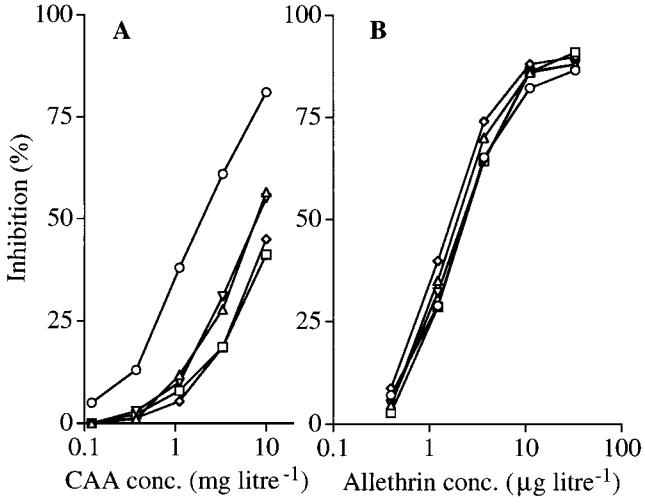

Fig. 3. The reactivity of KCA226 with (A) CAA or (B) allethrin under various pH conditions: (○) pH 5·0, (△) pH 6·0, (▽) pH 7·0, (□) pH 8·0 and (◇) pH 9·0. Results are represented as means of three experiments.

The reactivities of four antibodies with allethrin were also assayed in C-ELISA containing 30% methanol. As shown in Fig. 2B, the reactivity of KCA226 showed a linear inhibition curve at concentrations ranging from 300 to 10000 μg litre^{-1}, although the reactivity was decreased about 600-fold as compared with that in 0·5% methanol. The IC_{50} value of allethrin for KCA226 was about 2000 μg litre^{-1}. On the other hand, the reactivities of the polyclonal antibodies and the monoclonal antibody KCA190 were reduced in 30% methanol.

Based on these results, the monoclonal antibody KCA226 was found to be the most reactive of the four antibodies with allethrin in C-ELISA under both 0·5% and 30% methanol conditions.

3.4 Cross-reactivity of KCA226 with pyrethroid compounds

The cross-reactivity of KCA226 with pyrethroid compounds containing the CAA moiety was compared in C-ELISA under 0·5% and 30% methanol conditions, as listed in Table 1.

In 0·5% methanol, KCA226 was highly reactive with allethrin, bioallethrin, pyrethrins and tetramethrin, followed by phenothrin and resmethrin. These pyrethroid compounds have the common CAA moiety. On the other hand, KCA226 showed weak reactivity with cyfluthrin, cypermethrin and permethrin which have the dichlorovinyl-CAA moiety. In addition, KCA226 did not react with deltamethrin having the dibromovinyl-CAA moiety, the other pyrethroid compounds without the CAA moiety and CAA itself.

Since KCA226 was highly reactive with allethrin, bioallethrin, pyrethrins and tetramethrin as compared with phenothrin and resmethrin among the pyrethroid compounds having the CAA moiety, the reactivity of KCA226 with the CAA moiety seemed to be affected by the alcohol moieties of the pyrethroid compounds.

In 30% methanol, KCA226 also showed reactivity with allethrin, bioallethrin, pyrethrins and tetramethrin, but not with the other compounds, although the reactivity was considerably lower than that with 0·5% methanol. CAA was also not reactive with KCA226 in 30% methanol.

Based on these results, KCA226 was found to be specific to the CAA moiety of the pyrethroid compounds. The reactivity of KCA226 with these pyrethroid compounds was much higher in 0·5% methanol than in 30% methanol.

3.5 Effects of pH on the reaction of KCA226 and CAA

Since CAA was used as the hapten to produce the antibodies, KCA226 should be reactive with CAA. CAA is

one of the major metabolites of pyrethroid compounds. The reactivity of KCA226 with CAA was therefore examined under different pH conditions, since the effects of the COOH group of CAA may be changed under the assay conditions.

As shown in Fig. 3A, the reactivity of KCA226 with CAA was higher at pH 5·0 but lower at pH 6·0 to 9·0. On the other hand, the reactivity of KCA226 with allethrin was almost constant at different pH values as shown in Fig. 3B.

Thus, the reactivity of KCA226 with CAA was found to be lower under pH conditions leading to COO^-. Since the reactivity of KCA226 with CAA was much less than that with allethrin, C-ELISA based on KCA226 is not likely to be influenced by CAA contained in pyrethroid residues.

4 DISCUSSION

We have prepared both polyclonal and monoclonal antibodies specific to the CAA moiety of pyrethroid insecticides by the use of the hapten CAA. Among the antibodies prepared, the monoclonal antibody KCA226 was highly reactive with the pyrethroid compounds having the CAA moiety. Thus CAA is a useful target for immunoassay of the pyrethroid insecticide group containing that common structure.

Many polyclonal- and monoclonal-antibody-based ELISA systems have been developed for the analysis of pesticides.[12–17] In particular, the use of monoclonal antibodies has steadily increased in such assays. It is likely that monoclonal antibodies have an advantage for reactivity with pesticides.[8,10,18–20] KCA226 showed much higher reactivity with allethrin under conditions of 0·5% and 30% methanol than did the polyclonal antibodies, although KCA226 was more sensitive to higher concentrations of methanol than were the polyclonal antibodies. This advantage of KCA226 might result from the scheme used to screen antibodies secreted by hybridomas, which initially selected antibodies specifically reacting with CAA residues of CAA-BSA and then selected from these antibodies reacting with pyrethrins, since allethrin has a closely related structure to pyrethrin I, which is one component of pyrethrins, as shown in Fig. 4.

KCA226 showed specific reactivity with the pyrethroid compounds having the common CAA moiety, although it was much less reactive with CAA. This suggested that not only the CAA moieties but also the alcohol moieties of pyrethroids were important for reaction with KCA226. However, reactivities differed with the structures of the alcohol moieties. The steric hindrance of the alcohol moiety of the pyrethroid may affect the reactivity with KCA226.

Although CAA was used as the hapten, KCA226 was hardly reactive with CAA at pH 8·0, but more reactive at pH 5·0. This suggested that the COOH group of CAA was important for reaction with KCA226. In addition, since the cross-reactivity of KCA226 with CAA was much less than that with allethrin, CAA may not affect the assay of pyrethroid compounds in C-ELISA with KCA226.

Analysis of pyrethroid compounds is mainly performed by gas chromatography and/or high performance liquid chromatography.[21,22] Since such analytical processes require many steps, they are not only labour-intensive but also time-consuming. Therefore, development of a sensitive, convenient and economical method was required for the analysis of pyrethroid residues in a large number of food and environmental samples. Based on the present study, the immunoassay based on KCA226 appears to be useful for such assay of pyrethroid residues with the common CAA moiety. In particular, C-ELISA based on KCA226 may have potential for the simplified assay of pyrethroid compounds.

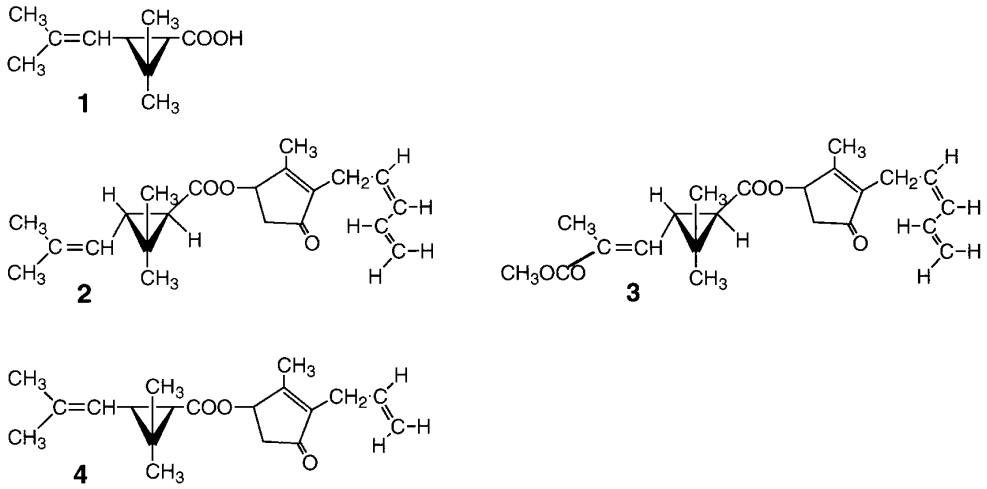

Fig. 4. The structures of compounds discussed: (**1**) chrysanthemic acid, (**2**) pyrethrin I (one main component of pyrethrins), (**3**) pyrethrin II (another main component of pyrethrins), (**4**) allethrin.

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